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Renal Localization and Persistence of Type 12 Streptococcal M-Protein.* (26990)

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The hypothesis that acute glomerulonephritis results from a hypersensitivity reaction has prompted investigations to explain why such reactions primarily involve the kidney. With respect to the possibility that the antigen might be a foreign protein, the association between Group A streptococcal infection, especially of certain types, *e.g.* Type 12, and the subsequent development of glomerulonephritis has focused attention on streptococcal substances.

Schmidt(1,2) reported that the Group A carbohydrate could be detected, using immunofluorescent techniques, in renal tubular cells of mice for up to 30 minutes following intravenous injection, and that a streptococcal protein could be demonstrated in cells of the reticulo-endothelial cells (RES) for as long as 60 hours. Kaplan(3) has extended the observations on the distribution of streptococcal proteins. He found that when small amounts of acid extracted M-protein were injected into mice, localization occurred largely in the phagocytic cells of the RES and within glomeruli. The M-protein was found to persist in glomeruli for at least 8 days, whereas outside the kidney virtually none was demonstrable by the fourth day.

On the basis of these observations, it might be postulated that the initial event in the pathogenesis of acute glomerulonephritis involves the deposition of M-protein within glomeruli and that subsequently, as circulating antibody against M-protein is produced, this antibody combines with the protein persisting in glomeruli, thereby damaging these structures. However, Kaplan detected no differences in the glomerular localization or persistence of M-protein from Types 1, 5, 12 and 19 Group A streptococci, so that even if the mechanism postulated above is true, the nephritogenic action of certain streptococcal types could not be explained simply on the basis of certain M-proteins having the special property of localizing and persisting in glomeruli.

Furthermore, the possibility exists that the distribution of M-protein observed by Kaplan reflects alterations in the protein produced during the process of its isolation. Thorbecke, Maurer and Benacerraf(4) showed that certain acid treated serum proteins may be rapidly cleared from the circulation by the kidney, whereas the unmodified protein is not.

The present investigation was undertaken to determine whether M-protein isolated with the use of a phage lysin would exhibit the same *in vivo* distribution as the material prepared by the relatively drastic procedure of hot acid extraction. The behavior of both preparations of M-protein was contrasted with that of acid denatured bovine serum albumin (BSA). It was found that phage prepared M-protein did localize and persist in glomeruli of mice, where it was demonstrated for as long as 16 days after a small intravenous injection.

Materials and methods. Animals: Swiss-Webster albino mice of both sexes, weighing 25-30 g were used.

Streptococci: Nass strain, Group A Type 12 streptococci, isolated during a focal outbreak of glomerulonephritis was generously

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provided by Dr. C. H. Rammelkamp, Jr. (Cleveland Metropolitan General Hosp., Cleveland, Ohio). The M-protein production of this strain was enhanced by repeated intraperitoneal passage in mice. Purity was frequently checked by plating, Gram staining, grouping, and typing. Strains of Types 1 and 3 were obtained from Dr. A. Bernheimer (New York Univ. School of Med.)

Acid denatured BSA was produced according to Maurer(5).

Antisera: Antisera specific for Type 12 were supplied by Dr. C. H. Rammelkamp, Jr. Antisera against other types were obtained from the U.S. Public Health Service Communicable Diseases Laboratory, Atlanta, Ga. Anti-whole streptococcal serum was prepared by repeated injection of washed heat and formalin killed bacteria into rabbits. The streptococci were grown in trypticase soy broth (Baltimore Biol. Lab., Baltimore, Md.). Antisera against acid denatured BSA were produced in rabbits(5). Sheep anti-rabbit gamma globulin antiserum was obtained through the courtesy of Dr. G. J. Thorbecke (New York Univ. School of Med.)

Fluorescein-conjugated antibody: Sheep anti-rabbit gamma globulin was conjugated with fluorescein using the isothiocyanate compound according to the method of Riggs *et al.*(6,7). Non-specific staining was completely eliminated by appropriate elution from a DEAE cellulose column(8) and by absorption with mouse tissue homogenates(3).

Fluorescent antibody techniques: Mice were killed by decapitation and pieces of lung, liver, spleen, and kidney were frozen by immersion in liquid nitrogen and stored at −22°C. until sectioned in a cryostat. Following sectioning, the tissue was fixed for 20 minutes in acetone. Sections were stained by the indirect technique, except for those experiments in which rabbit gamma globulin was looked for and in this case the direct technique using fluorescein-conjugated sheep anti-rabbit gamma globulin was employed. The following observations were considered to demonstrate the specificity of positive staining: 1) failure to stain normal mouse tissues; 2) failure to stain smears of Types 1 or 3 streptococci; 3) failure to stain tissues of mice injected with Type 12 M-protein using antisera directed against Types 1, 3, 19, or 25, or Group A polysaccharide; and 4) failure to stain tissues of experimental animals with Type 12 antiserum which had been absorbed with Type 12 M-protein(3). Tissues were also fixed in buffered formalin and hematoxylin and eosin stained sections were prepared.

M-protein: M-protein was prepared by 2 methods. Acid extracted M-protein, Type 12, was prepared from washed bacterial cell walls(9) according to the method described by Lancefield and Perlmann(10). M-protein was also prepared from cell walls by the use of a lysin associated with streptococcal bacteriophage(11). This work was done in collaboration with Dr. Richard M. Krause (Rockefeller Inst. New York). In both cases M-protein was precipitated by 0.33-0.6 saturated ammonium sulfate. All fractions were tested for precipitin reactions by double diffusion in agar(12) and immunolelectrophoresis(13) against typing, grouping, and anti-whole streptococcal antisera. Both preparations showed strong single lines against the anti-Type 12 M sera but not against the other typing sera. Untreated preparations of both materials gave no reaction with antiserum against the Group A polysaccharide, but when the lysin prepared M-protein was first treated with *Streptomyces albus* enzyme, a proteolysin, a faint line was evident(11). With both acid and phage prepared M-protein, the anti-whole streptococcal serum gave a very strong single line, corresponding to M and several very much fainter lines. Protein content was determined by Biuret and microKjeldahl methods.

Experimental. Mice were divided into 7 groups as follows:

Group I consisted of normal untreated animals.

Group II consisted of 8 mice given 0.5 mg of acid extracted M-protein and 25 mice given 1.0 mg of acid-extracted M-protein. Material in this group, and in those described

TABLE I. the Persistence of Group A Type 12 Streptococcal M-Protein and Acid Denatured BSA in Glomeruli of Mice After I.V. Injection.

	Material injected					
	Acid extracted M		Phage Lysin prepared M		Denatured BSA	
Day killed post-injection	Dose (mg) .5	1.0	Dose (mg) .5	1.0	Dose (mg) .5	1.0
1	1/1*	1/1	1/1		1/1	
2	1/1		1/1		1/1	
3	1/1		1/1		1/1	2/2
4	1/1	2/3	1/1	3/3	0/1	1/2
5	1/1	3/3	1/1	2/2	0/1	
6	1/1		1/1		0/1	
7	1/1		0/1		0/1	
8	1/1	2/2	1/1	2/2	0/1	0/2
9		1/2		2/2		
12		3/4	1/3	2/3		
16		2/3		2/3		
20		0/4		1/3		
25		0/3		0/3		

* Indicates number of animals with glomeruli containing detectable amounts of M-protein or acid denatured BSA over number of mice studied.

below was administered intravenously in the tail vein in a volume of 0.5 ml.

Group III consisted of 11 mice given 0.5 mg and 21 mice given 1.0 mg of phage lysin prepared M-protein.

Group IV consisted of 8 mice given 0.5 mg and 6 mice given 1.0 mg of acid denatured BSA.

All of the animals in the above groups were killed on various days following injection according to the schedule shown in Table I.

Groups V, VI and VII consisted of 6 mice each. Animals in these 3 groups were given 1.0 mg of acid extracted M-protein, phage lysin prepared M-protein and acid denatured BSA respectively. One day later the animals in each group were given an intravenous injection of 0.5 mg of the appropriate antibody protein intravenously. Three of the mice in each group were killed 2 days later and the remaining mice 3 days later.

Organs from all animals were examined by fluorescent and light microscopy.

All of the animals given either acid extracted or phage lysin prepared M-protein alone showed distribution and persistence patterns essentially the same as those described by Kaplan(3). Fluorescent material was present in the phagocytic cells of the liver, spleen, and to a lesser extent, of the lungs for the first 2 days following injection. M-protein was not apparent in any other location examined, outside of the kidney, including hepatic parenchymal cells, alveolar walls or pulmonary capillaries, or in the germinal centers of the spleen. By the third day only traces could be detected in liver or spleen and none was apparent in the lung. At 4 days and later no fluorescent material was demonstrated except in glomeruli. There appeared to be no difference between animals given 0.5 or 1.0 mg.

The picture in the kidneys was quite different. Throughout this period M-protein was evident in high concentration in almost all glomeruli following administration of either preparation. Only rare deposits were noted in the interstitial tissues. The only difference observed between the 2 materials occurred during the first 2 or 3 days following administration, when the phage lysin prepared material showed a moderate amount of tubular deposition, fluorescence being present in nuclei, cytoplasm and tubular lumens. This staining was not seen after the fourth day.

Staining in individual glomeruli characteristically consisted of small aggregates of fluorescent material that at times assumed a globular configuration. While the M-protein appeared to be deposited intracellularly it was impossible to determine if capillary endothelial cells or epithelial cells were principally

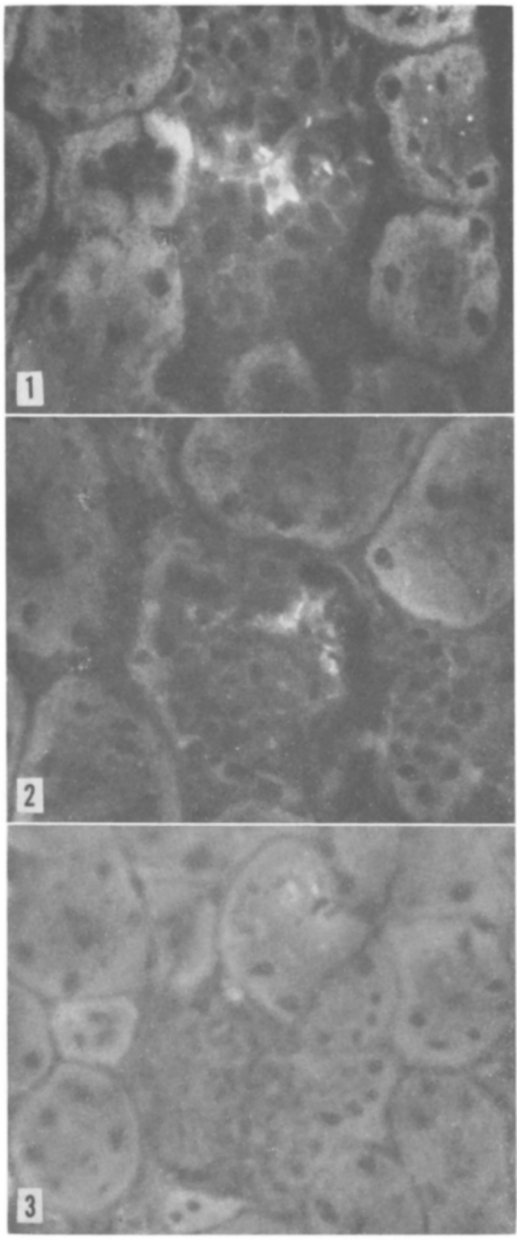

FIG. 1. Type 12 M-protein (phage lysin prepared) in mouse glomerulus 4 days after injection of 1.0 mg I.V. Stained with rabbit anti-Type 12 M-protein antisera followed by fluorescein conjugated sheep anti-rabbit gamma globulin antibody (528 ×).

FIG. 2. Phage lysin prepared Type 12 streptococcal M-protein in mouse glomerulus 16 days after injection of 1.0 mg I.V. Stained as in Fig. 1 (528 ×).

FIG. 3. Normal mouse glomerulus (528 ×).

involved. Nuclei of involved cells did not stain (Fig. 1).

During the first 4 days the amount of M-protein, using either acid extracted or phage lysin prepared material, in the kidney seemed to decrease as evidenced by a diminution in the extent of staining within individual glomeruli. Throughout this period M-protein was readily detectable in the urine by means of capillary precipitin tests. From the fourth to the eighth day after administration there appeared to be little change and the great majority of glomeruli continued to show deposits. Between days 8 and 12 the number of involved glomeruli decreased considerably but fluorescence could still be seen in many glomeruli at the end of this time. By the sixteenth day only occasional glomeruli showed M-protein (Fig. 2). In these the fluorescence was quite bright although not extensive. With one exception no positive results were obtained beyond 16 days.

Of the animals given M-protein alone, only 3 showed evidence of pathologic changes in the form of equivocal focal inflammation in glomeruli. Two of these mice had been given 1.0 mg of phage lysin prepared material and one animal had been given 1.0 mg of acid extracted M-protein; all were killed 16 days after injection.

In Group IV, in which animals were given acid denatured BSA, during the first 2 or 3 days only small amounts of acid denatured BSA could be detected in cells of the RES, glomeruli or renal tubular cells. Large amounts of the modified BSA were readily detectable in the urine as determined by precipitin reactions with specific antibody. By the fourth day no fluorescence was observed in any of the tissues of any animal in this group. No pathologic changes were noted.

No lesions appeared in the kidneys or other organs of those animals given antibody after receiving either M-protein or denatured BSA. Fluorescent microscopy revealed only very small amounts of rabbit gamma globulin, presumably specific antibody, in the glomeruli. Animals which received the acid denatured BSA and antibody directed against it showed considerably less renal deposition of the antibody than animals given the M-protein system.

Discussion. The relationship between

streptococcal infection and acute hemorrhagic glomerulonephritis in man has led to a consideration of various streptococcal substances as either participants in an immune reaction within glomeruli or as substances capable of directly damaging glomeruli(14).

The association between nephritis and certain types of Group A streptococci warrants consideration of the possibility that the type specific M-protein plays a role in the pathogenesis of the renal damage. It appears, in view our findings, that M-protein possesses the property of localizing and persisting in the glomeruli of mice for many days. This property does not seem to be the result of alterations in the protein occurring during its isolation, since there was no apparent difference in the behavior of either M-protein preparation.

The ability of M-protein to localize and persist in glomeruli for long periods of time renders plausible the hypothesis that this streptococcal antigen participates in pathogenesis of acute nephritis on an immune basis. Such a period of time is more than the minimal limit required for production of antibody, and that this material can be detected by immunofluorescent methods indicates that it has retained its capacity to combine with antibody during this interval. The demonstration that circulating antibody, even in small amounts, has access to the antigen for at least 4 days after its administration further emphasizes this point. The failure to produce glomerular lesions by administration of antibody after injection of M-protein under the conditions employed here does not support this hypothesis, but different schedules or dosages might lead to the production of lesions.

While only equivocal glomerular abnormalities were observed in a small number of the animals injected with M-protein alone it is possible that administration of larger amounts, greater than 1 mg, might produce lesions. In this connection the possibility should be noted that M-protein might possess certain tissue damaging properties, especially in the case of the lysin preparation. Cromartie and his associates(15) have observed that a polysaccharide-protein complex obtained from Group A streptococci is toxic for rabbit connective tissue. Krause has provided evidence for a similarity between this substance and phage lysin prepared M-protein by demonstrating that the latter is associated with a polysaccharide moiety(11).

Summary. The findings of Kaplan with regard to the renal localization of Type 12 streptococcal M-protein have been confirmed and extended. It has been shown that this property is not the result of denaturation during preparation. The significance of these findings in regard to hypotheses concerning the pathogenesis of acute nephritis is briefly discussed.

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